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High-Speed Quantitative UPLC-MS Analysis of Multiple Amines in Human Plasma and Serum via Precolumn Derivatization with 6-Aminoquinolyl-*N*-hydroxysuccinimidyl Carbamate: Application to Acetaminophen-Induced Liver Failure

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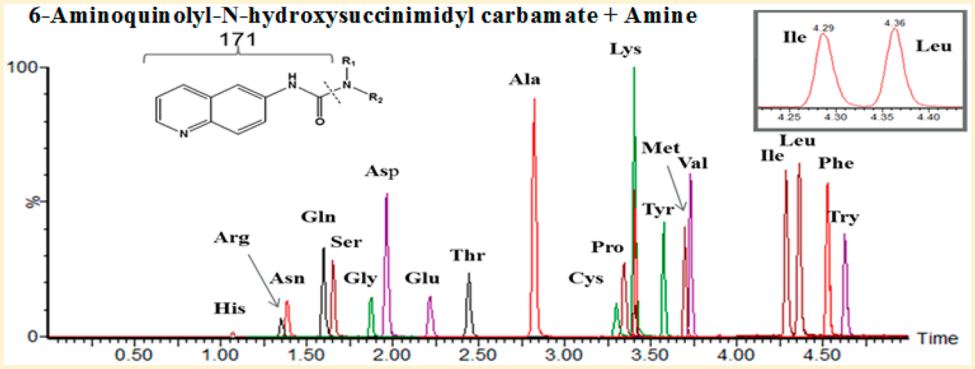
Supporting Information



ABSTRACT: A targeted reversed-phase gradient UPLC-MS/MS assay has been developed for the quantification /monitoring of 66 amino acids and amino-containing compounds in human plasma and serum using precolumn derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AccQTag Ultra). Derivatization of the target amines required minimal sample preparation and resulted in analytes with excellent chromatographic and mass spectrometric detection properties. The resulting method, which requires only 10 μL of sample, provides the reproducible and robust separation of 66 analytes in 7.5 min, including baseline resolution of isomers such as leucine and isoleucine. The assay has been validated for the quantification of 33 amino compounds (predominantly amino acids) over a concentration range from 2 to 20 and 800 μM. Intra- and interday accuracy of between 0.05 and 15.6 and 0.78−13.7% and precision between 0.91 and 16.9% and 2.12−15.9% were obtained. A further 33 biogenic amines can be monitored in samples for relative changes in concentration rather than quantification. Application of the assay to samples derived from healthy controls and patients suffering from acetaminophen (APAP, paracetamol)-induced acute liver failure (ALF) showed significant differences in the amounts of aromatic and branched chain amino acids between the groups as well as a number of other analytes, including the novel observation of increased concentrations of sarcosine in ALF patients. The properties of the developed assay, including short analysis time, make it suitable for high-throughput targeted UPLC-ESI-MS/MS metabonomic analysis in clinical and epidemiological environments.

Untargeted metabolic phenotyping (metabotyping[1,2]) as performed in metabonomic/metabolomic studies offers the possibility of discovering new biomarkers.[3,4] The use of LC-MS-based techniques for this purpose is now widespread[5−7] with an increasingly role evident in biomarker discovery in large-scale epidemiological and personalized medicine studies (e.g.[8−12]). However, as is widely appreciated, untargeted methods generally provide relative changes (fold changes) for metabolites, rather than absolute concentration data. In addition, despite the application of, for example, high-resolution UHPLC separations and the use of combinations of separation techniques (particularly reversed-phase and HILIC modes of chromatography[12]) for sample analysis, the coverage of the metabolome remains far from comprehensive. A consequence of this partial, and qualitative, coverage is there is a need for

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subsequent further validation of the changes detected by untargeted methods. For example, a recent study of plasma amino acid profiles in renal transplant patients compared the results obtained using both an untargeted metabolic profiling method against a specific LC-MS/MS assay showing that, while there was overlap between the data obtained by both methods, there were also significant differences.[13] Use of a validated targeted assay, based on optimized sample preparation and bespoke LC-MS conditions, combined with appropriate internal standards, enables quantitative data to be obtained for the analytes of interest, which is especially valuable in clinical biomarker discovery. In addition, compounds related to the target analytes that may not have been detected in the original metabotyping study can also be determined. Amino acids, and amino-containing compounds, are modulated in many different conditions (e.g., toxicity, cancer, metabolic diseases, etc.) and often vary in epidemiological or clinical metabolic phenotyping studies. As such, amino compounds represent an obvious class of compounds for targeted analysis. There are of course innumerable methods for the analysis of complex mixtures of amino acids and biogenic amines (dating back to the pioneering work of, e.g., Martin[14] and Dent[15] and their co-workers using 2D paper chromatography). Currently many quantitative methods for amino compounds use MS for detection (reviewed in refs 16,17) including those based on e.g., GC-MS,[18,19] CE-MS/MS,[20,21] and LC-MS/MS.[22−44] However, for reversed-phase (RP) LC-based methods, the amphoteric nature of the amino acids often results in poor retention, making direct analysis impractical for all but a few analytes. Alternative modes of LC that improve retention allowing direct analysis include separations based on strong cation exchange,[22] HILIC,[23,24] or ion-pairing.[25−32] However, such approaches are not without disadvantages and, as a result, many methods rely on forming derivatives of amino compounds that enable RPLC to be performed. A number of reagents are available for the derivatization of amino acids to facilitate analysis by LC-MS.[33−44] One of these, 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AccQTag Ultra), originally applied to the determination of primary and secondary amines via fluorescence detection, for example,[45−49] has also been employed for sensitive and specific LC-MS detection.[41−44] Here a high-throughput, sensitive, and selective UPLC-ESI-MS/MS assay for the targeted analysis of amino acids and biogenic amines in human plasma or serum based on the AccQTag Ultra reagent is described. This method forms one of a suite of targeted[50,51] and untargeted[12] methods developed, or under development, to support metabolic phenotyping and biomarker validation for the MRC-NIHR National Phenome Centre. The method was applied to samples obtained from healthy controls and patients suffering from acute liver failure (ALF) resulting from acetaminophen (APAP, paracetamol) overdose.

EXPERIMENTAL SECTION

Chemicals and Reagents. Analyte standards (listed in Table 1) were from Sigma-Aldrich (Gillingham, U.K.). Isotopically labeled amino acids for use as internal standards (IS) (see Table 1) were from Cambridge Isotope Laboratories (MA, U.S.A.) or QMX Laboratories (Essex, U.K.) (see Table S9 caption for details). Optima grade water was obtained from Fisher Scientific (Leicester, U.K.), LC-MS grade solvents and formic acid were from Sigma-Aldrich (Gillingham, U.K.) and the AccQTag Ultra reagent from Waters Corporation (Milford, MA, U.S.A.).

Samples. Plasma samples were obtained from 14 patients with acetaminophen-induced acute liver failure (ALF) (2 male, 12 female; 19 to 56 (mean = 38) years of age) and 40 healthy volunteers (20 male, 20 female; 32−41 (mean = 37) years of age) as controls. A Mann U Whitney test found that the ages of the subjects in the control and APAP-induced ALF groups were well matched (p-value = 0.2103). Local national research ethics service (NRES) approval was obtained for this study and patients, or their nominee, provided written informed consent within 24 h of admission to Kings College Hospital London. Blood samples were obtained within 24 h of admission into BD Vacutainer lithium heparin-containing vacuum tubes (Franklin Lakes, NJ). Plasma was obtained by centrifugation (12 000*g*, 4 °C, 10 min.) within 1 h of sample collection and was then stored at −80 °C.

Analytical Procedure. *Preparation of Stock Solutions.* Calibration and QC samples were prepared from a standard mixture of neutral, basic, and acidic physiological amino acids (Sigma-Aldrich) with the addition of asparagine and glutamine on each day of the validation. Duplicate working stock solutions (A and B) were made in 50:50 water/methanol (v/v) at a concentration of 400 μM for each analyte. Dilutions of stock A were used to prepare calibration standards and dilutions of stock B for QC samples. For validation, 16 compounds were quantified against stable-isotope-labeled internal standards with 17 validated using a surrogate internal standard (see Table 1).

Calibration, Quality Control (QC), and Stable-Isotope-Labeled (SIL) IS Solutions. Calibration standards were prepared by dilution with 50:50 water/methanol (v/v) to give concentrations of 0, 1, 2, 4, 10, 20, 40, 100, 200, and 400 μM. QC samples were prepared by dilution with 50:50 water/methanol (v/v) to provide concentrations at the lower limit of quantification (LLOQ) (1, 3, or 10 μM depending upon the analyte), low-level (3, 10, or 30 μM depending upon the analyte), midlevel QC (30 or 150 μM depending on the analyte), high-level (300 μM), and upper limit of quantification (400 μM) of each amino acid. Solutions of each SIL amino acid (1 mg/mL) in Optima grade water were combined to provide a stock solution at a concentration of 10 μg/mL. A 20 μL aliquot of each calibration and QC standard were transferred to an Eppendorf tube followed by 5 μL of the IS solution (IS was not added to the blanks), and 40 μL of cold isopropanol (IPA) containing 1% formic acid (v/v). After 20 min at −20 °C samples were centrifuged (13 000*g*, 10 min) and 10 μL of supernatant transferred to a glass HPLC vial (or 96-well plate) for derivatization.

Sample Preparation. Human plasma or serum samples were left to thaw at 4 °C, and then 10 μL of each sample was transferred to an Eppendorf tube to which 10 μL of Optima grade water was added then 5 μL of the 10 μg/mL IS mixture. Proteins were then precipitated using 40 μL of cold isopropanol (plus 1% formic acid (v/v)) with vortex mixing. After 20 min at −20 °C, samples were centrifuged 13 000*g*, 10 min), and then 10 μL of the supernatant was transferred to a glass HPLC vial (or 96-well plate) for derivatization as described below.

Derivatization. For derivatization, 1 mL of acetonitrile was added to the AccQTag Ultra reagent powder, vortex mixed, and dissolved by heating at 55 °C (no longer than 15 min). Then, 70 μL of borate buffer (pH 8.6) was added to the samples (with vortex mixing) followed by 20 μL of AccQTag Ultra derivatizing reagent solution, with further vortex mixing, and heating at 55 °C (10 min). Samples were then diluted 1:100

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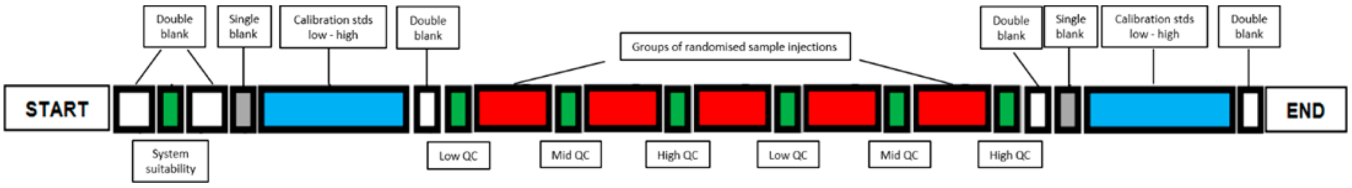

Figure 1. Sequence of analysis for amino acid quantification of randomized samples bracketed by calibration standards and interspersed with QC injections.

with Optima grade water for analysis. Sample preparation is summarized in Supplementary Table S1

UHPLC-MS/MS Analyses. UHPLC-MS/MS analysis was performed using an Acquity UPLC binary solvent manager, sampler manager, and column manager (Waters, Milford, MA, U.S.A.) interfaced with a Xevo TQ-S tandem quadrupole mass spectrometer (Waters, Wilmslow, U.K.). MS/MS Detection was via electrospray ionization (ESI) in positive ion mode using multiple reaction monitoring (MRM) for the quantification of each compound (see Table 1) (MS conditions for each analyte were determined via direct infusion of individual derivatives). Nitrogen was used as the desolvation gas, and argon was used as the collision gas. The following generic source conditions were used: capillary voltage, 1.5 kV; source offset, 50 V; desolvation temperature, 600 °C; source temperature, 150 °C, desolvation gas flow, 1000 L/h; cone gas flow, 150 L/h; nebulizer gas, 7.0 bar; collision gas, 0.15 mL/min. Compound-specific parameters are detailed in Table 1.

The chromatographic separation used reversed-phase gradient chromatography on a HSS T3 2.1 × 150 mm, 1.8 μm column (Waters). The mobile phase was composed of 0.1% formic acid in water (v/v) (A) and 0.1% formic acid in acetonitrile (v/v) (B). The column temperature was maintained at 45 °C and linear gradient elution was performed at 0.6 mL/min starting at 4% B, held for 0.5 min before increasing to 10% over 2 min, then to 28% over 2.5 min, and finally increasing to 95% for 1 min, before returning to 4% B (1.3 min) for re-equilibration. The weak and the strong washes were 95:5 water/acetonitrile (v/v) and 100% isopropanol, respectively.

Before analysis, injections (2 μL) of a double blank were performed to ensure system stability and cleanliness followed by a "system suitability test", performed by injecting the low-level QC sample containing all the standards and internal standards. After the injection of a further double blank and a single blank, analysis was started with injections of the calibration curve (low concentrations to high) followed by a double blank injection. The QC standards (at least 6 QC samples, 2 at each level) were interspersed evenly throughout the study samples as shown in Figure 1. Study samples were randomized prior to sample preparation to minimize bias due to batch effects. Following analysis of all of the samples in the batch, a second set of calibration samples were injected, again using the sequence of low to high concentrations.

Method Validation. Method validation for the method was based as far as practicable on the FDA "Guidance for Industry" on Bioanalytical methods.[52]

Intra- and Inter-Assay Precision. To determine assay precision calibration standards prepared from stock A and 6-fold replicates from stock B at the same concentration as six of the calibration standards were analyzed as QC samples, in a single batch using the methods described. Linearity was assessed using the R^2 correlation coefficient determined from calibration standards was required to be >0.99 over the 3 days of the validation.

The intra-assay variability of the method was determined using the CV for replicate assays (n = 6) for each of the six selected concentrations on a single occasion. Interassay precision was assessed on three separate days using six QC samples at three concentrations assayed together with a set of calibration standards and biological samples and determined as the CV for each set of the QC samples (n = 18).[52] To be accepted, a minimum of 67% of the QC standards must have had a deviation of no more than 15% from their nominal concentration, with at least 50% of the QC injections at each concentration meeting this criterion.

Specificity. Human plasma and serum from six different subjects were tested to determine matrix interferences using stable-isotope-labeled analogues.

Carryover. Carryover was assessed with a double blank run immediately after an ULOQ calibration standard and was accepted if the response was ≤20% of the average response from the LLOQ standards. Carryover for the IS was acceptable if the response in the double blank sample that was ≤5% of the average response from the calibration standards (including the single blank).

Recovery. In the absence of analyte free matrix, recovery from plasma/serum from six sources was estimated using stable-isotope-labeled (SIL) compounds. Recovery was calculated by comparing the responses for six replicates of extracted samples spiked at 1 μg/mL (0.24−0.67 μM depending on the analyte) and at 3 μM, with replicates of extracted blank matrix to which SIL(s) were added postextraction, at the same nominal concentrations.

Matrix Interferences. Matrix-to-analyte interferences were assessed by analyzing six double blanks for responses at the retention times of the analytes compared to the mean of the analyte responses in the LLOQ calibration standards. A minimum of five of the six double blanks had to be less than 20% of the signal in the LLOQ calibration standard. Matrix effects on the SIL ISs were assessed using the same approach with the acceptance criteria based on signals being less than 5% of the average IS response of the standards in the calibration curve in at least five of the six double blanks. For analyte to analyte interference aliquots of the same lot of blank matrix were spiked with each analyte, in triplicate, at the same concentration as the highest concentration standard and analyzed to assess their potential interference at the retention time of the other analytes. Interference was considered to be present if a response ≥20% to that of another analyte was detected for the LLOQ standard. For analyte to IS interference, the blank matrix was spiked with analytes at the concentration of the highest concentration standard and responses at the retention time of the IS were compared to the average response of the IS of all standards accepted in the calibration curve

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Table 1. Chromatographic and Mass Spectrometric Characteristics of the Analytes[a]

no. compound	parent (m/z)	fragment (m/z)	RT (min)	dwell time (s)	cone voltage (V)	collision energy (eV)	LLOQ (μM)[a]
1 *creatinine*	227.0	158.9	0.51	0.024	30	6	-
2 *creatine*	302.1	112.1	0.61	0.024	62	20	-
3 *galactosamine*	350.1	171.1	0.81	0.024	30	32	-
A histidine-d3	329.1	159.1	1.53	0.011	20	10	n/a
4 histidine	326.1	156.1	1.55	0.011	20	10	6
5 *N-methyl-L-phenylalanine*	351.1	207.0	1.56	0.013	30	24	n/q
6 phosphoserine*A	356.1	171.1	1.61	0.013	20	18	n/q
7 *methylamine*	202.1	171.1	1.63	0.010	30	16	-
8 3-methylhistidine*A	340.1	170.1	1.73	0.009	30	18	20
9 4-hydroxyproline*C	302.0	171.1	1.75	0.011	10	22	2
10 1-methylhistidine*A	340.1	124.2	1.84	0.009	30	28	20
11 asparagine*D	303.1	171.1	1.97	0.009	30	22	2
12 carnosine*C	397.1	227.2	1.99	0.009	30	14	20
13 arginine	345.1	70.1	2.09	0.009	30	36	20
14 *anserine*	411.2	241.1	2.03	0.009	68	18	-
B arginine-13C6	351.2	181.2	2.15	0.009	6	18	n/a
15 *glycylglycine*	303.1	171.1	2.15	0.009	30	22	-
16 taurine*B	296.1	116.3	2.18	0.009	30	60	n/q
C glutamine-d5	322.1	171.1	2.26	0.009	30	24	n/a
17 glutamine	317.1	171.1	2.26	0.009	30	24	2
D serine-d3	279.1	171.1	2.28	0.009	30	20	n/a
18 serine	276.1	171.1	2.28	0.009	30	20	6
19 homoserine*C	290.1	171.1	2.42	0.013	10	18	n/q
20 ethanolamine*D	232.1	171.1	2.43	0.011	10	20	2
E glycine-d5	248.1	171.0	2.56	0.011	30	20	n/a
21 glycine	246.1	116.1	2.56	0.011	30	40	20
22 aspartic acid	304.1	171.1	2.65	0.011	30	22	2
F aspartic acid-d3	307.0	171.1	2.66	0.011	30	20	n/a
23 citrulline*D	346.2	171.1	2.75	0.011	30	26	6
24 sarcosine*F	260.1	171.1	2.82	0.011	30	20	2
G glutamic acid-d3	321.0	171.0	2.93	0.011	30	20	n/a
25 glutamic acid	318.1	171.1	2.94	0.011	30	22	2
26 3-aminopropanoic acid*G	260.1	171.1	3.04	0.011	30	20	n/q
27 threonine	290.1	171.1	3.07	0.018	30	20	2
H threonine-13C4,15N	295.1	171.0	3.13	0.024	30	26	n/a
28 *ophthalmic acid*	460.3	171.0	3.40	0.011	48	36	-
I alanine-d3	263.1	171.0	3.43	0.011	30	16	n/a
29 alanine	260.1	116.1	3.43	0.011	30	44	2
30 *allantoin*	329.0	171.1	3.43	0.011	30	20	-
31 4-aminobutyric acid (GABA)*J	274.1	171.1	3.50	0.009	10	20	6
32 aminoadipic acid*J	332.1	171.1	3.59	0.011	30	18	-
33 hydroxylysine*J	333.2	171.1	3.50	0.009	16	16	20
34 3-aminobutyric acid	274.1	171.1	3.67	0.009	10	20	6
35 *cysteine*	292.1	171.1	3.75	0.009	30	22	-
36 *glutathione*	478.0	171.1	3.76	0.009	30	38	-
J proline-d7	293.0	171.1	3.76	0.009	30	24	n/a
37 proline	286.1	116.1	3.79	0.009	30	50	6
38 3-aminoisobutyric acid*J	274.1	171.1	3.77	0.009	10	20	
39 cystathionine*J	282.2	171.0	3.85	0.009	30	14	2
40 ornithine*J	303.1	171.1	3.86	0.009	60	22	20
41 *epinephrine*	354.1	171.1	3.93	0.009	30	22	-
42 2-aminoisobutyric acid	274.1	171.1	3.97	0.009	10	20	6
43 *5-aminovaleric acid*	288.1	171.1	3.98	0.009	30	16	-
44 *octopamine*	324.2	171.1	3.98	0.009	30	24	-
45 2-aminobutyric acid*K	274.1	171.1	4.07	0.009	10	20	6
46 cystine*K	291.1	171.1	4.08	0.009	10	12	n/q
K lysine-d4	246.1	171.1	4.10	0.020	30	14	n/a
47 lysine	244.2	171.1	4.10	0.020	30	12	2
48 *putrescine*	429.3	145.1	4.22	0.009	30	28	-
49 *4-aminohippuric acid*	365.2	171.0	4.36	0.019	6	36	-
50 *3-hydroxytyramine*	324.1	171.1	4.41	0.008	30	20	-

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Table 1. continued

no. compound	parent (m/z)	fragment (m/z)	RT (min)	dwell time (s)	cone voltage (V)	collision energy (eV)	LLOQ (μM)[a]
51 tyrosine*M	352.1	171.1	4.41	0.017	30	24	2
52 *3-hydroxykynurenic acid*	395.2	171.0	4.57	0.009	60	28	-
L methionine-13C5,15N	326.2	171.0	4.59	0.017	30	22	n/a
53 methionine	320.1	171.1	4.59	0.017	30	22	2
M valine-d8	296.1	171.1	4.61	0.017	30	20	n/a
54 *homocysteine*	306.0	171.1	4.62	0.017	30	20	-
55 valine	288.1	171.1	4.64	0.017	30	16	2
56 *tyramine*	308.1	171.1	4.82	0.017	30	24	-
57 *N-methyl-L-valine*	302.0	171.1	4.84	0.017	54	26	-
58 *homocystine*	439.1	171.1	4.87	0.009	10	24	-
59 *3-hydroxyanthranilic acid*	324.2	171.1	4.88	0.017	52	20	-
N isoleucine-d10	312.1	171.0	5.16	0.024	30	20	n/a
60 isoleucine	302.1	171.1	5.22	0.024	30	20	2
O leucine-d10	312.1	171.0	5.26	0.024	30	20	n/a
61 *O-phosphoethanolamine*	312.1	116.1	5.26	0.024	8	50	-
62 leucine	302.1	171.1	5.26	0.024	30	20	2
P phenylalanine-d5	341.1	171.1	5.44	0.024	30	22	n/a
63 phenylalanine	336.1	171.1	5.46	0.024	30	22	2
64 tryptophan*P	375.1	171.1	5.51	0.024	30	26	2
65 *tryptamine*	331.1	171.1	5.86	0.069	30	28	-

[a]Normal font − validated against stable-isotope-labeled IS of the same structure; normal font* − validated against a stable-isotope-labeled IS of a different structure (indicated by letter after *) but similar retention time; italic font − not validated but monitored; bold font − stable-isotope-labeled IS. [b]Based on a 10 μL plasma or serum sample.

(including single blank(s)). Interference was considered to be present if a response ≥5.0%.

Stability. Stock solution stability of the underivatized analytes at ambient temperature (6 h) or stored frozen at −20 °C (48 h) was investigated. The stability of the derivatized analytes (diluted 1:9 v/v with water) was also assessed by storing the QC samples at ambient temperature (1 week) and in the autosampler (diluted 1:99 v/v with water) and by reanalysis of the QC samples maintained in the autosampler at 4 °C (36 h). For the analyte to be considered stable the difference had to be within ±10% of the original value.

Data Analysis. The raw LC-MS data were processed by the TargetLynx application package within MassLynx software (Waters Corporation). The raw data was mean smoothed, and peak integration was performed using ApexTrak algorithm. Further statistical analysis was performed on the resulting calculated concentrations (corrected for the 2-fold dilution of the samples compared to the standard curve) using Prism, where a Mann U Whitney test was applied to determine if differences observed in concentrations between healthy volunteers and patients with ALF were statistically significant.

RESULTS AND DISCUSSION

Method Development. The AccQTag Ultra reagent reacts with primary and secondary amines[44−49] (illustrated in supplementary Figure S1) to give derivatives with good reversed-phase chromatographic and mass spectrometric properties. Potential problems associated with the use of the nonvolatile borate buffer combined with MS have previously been discussed, and volatile alternative buffers have been evaluated.[43] However, probably as a result of large dilution factor employed here, we encountered no buffer-related problems, and no changes were made to the recommended reaction conditions. Appropriate positive ESI MS conditions for each of the analytes were obtained via direct infusion of the individual derivatives, as detailed in Table 1 (columns 3, 4, 6, 7, 8 respectively). The AccQTag Ultra reagent gives rise to a common fragment ion at m/z 171, generated by a loss of the aminoquinoline (AMQ) moiety, and as can be seen from Table 1 (columns 3 and 4), in most cases, the combination of parent ion and this common fragment was selected for detection. While monoamines react to form a mono ACQ derivative which ionizes in positive ESI to the $[M+Acq+H]^+$ ion, some polyamines (e.g., cystine, lysine) have multiple sites that can form derivatives resulting in the addition of more than one ACQ unit. The number of ACQ units is reflected in the charge state of the analyte, and doubly derivatized compounds display the most intense response. However, a smaller proportion of the singly derivatized and singly charged and doubly derivatized and singly charged ions are still detected. For example, for cystine and lysine, the most abundant ions have m/z values of 291 and 244, respectively, corresponding to the $[M+2xAcq+2H]^{2+}$, while ions at m/z values of 581 ($[M+2xAcq+H]^+$) and 411 ($[M+1xAcq+H]^+$) for cystine and 487 ($[M+2xAcq+H]^+$) and 317 ($[M+1xAcq+H]^+$) for lysine were observed at lower abundances (for mass spectra, see supplementary Figure S2). For quantification, the most abundant transitions from each of these precursor ions were used except for arginine, glycine and proline where a more appropriate alternative transition was selected due to matrix interferences. A chromatographic method was then developed providing an analysis time of 7.5 min/sample (a mass chromatogram for a range of amino compound standards is shown in Figure S3 and a similar mass chromatogram for the isotopically labeled internal standards is shown in Figure S4). The average peak width observed was 3 s at the base giving a peak capacity of ca.120. The separation was highly reproducible, with CVs for retention time (Table 1 column 5) for the ULOQ QC sample of <0.44%.

A number of the compounds determined in this assay are isomers/isobars, for example, 1-methylhistidine and 3-methylhistidine; sarcosine, 3-aminopropanoic acid (β-alanine), and alanine; 4-aminobutyric acid (GABA) acid, 2-aminoisobutyric

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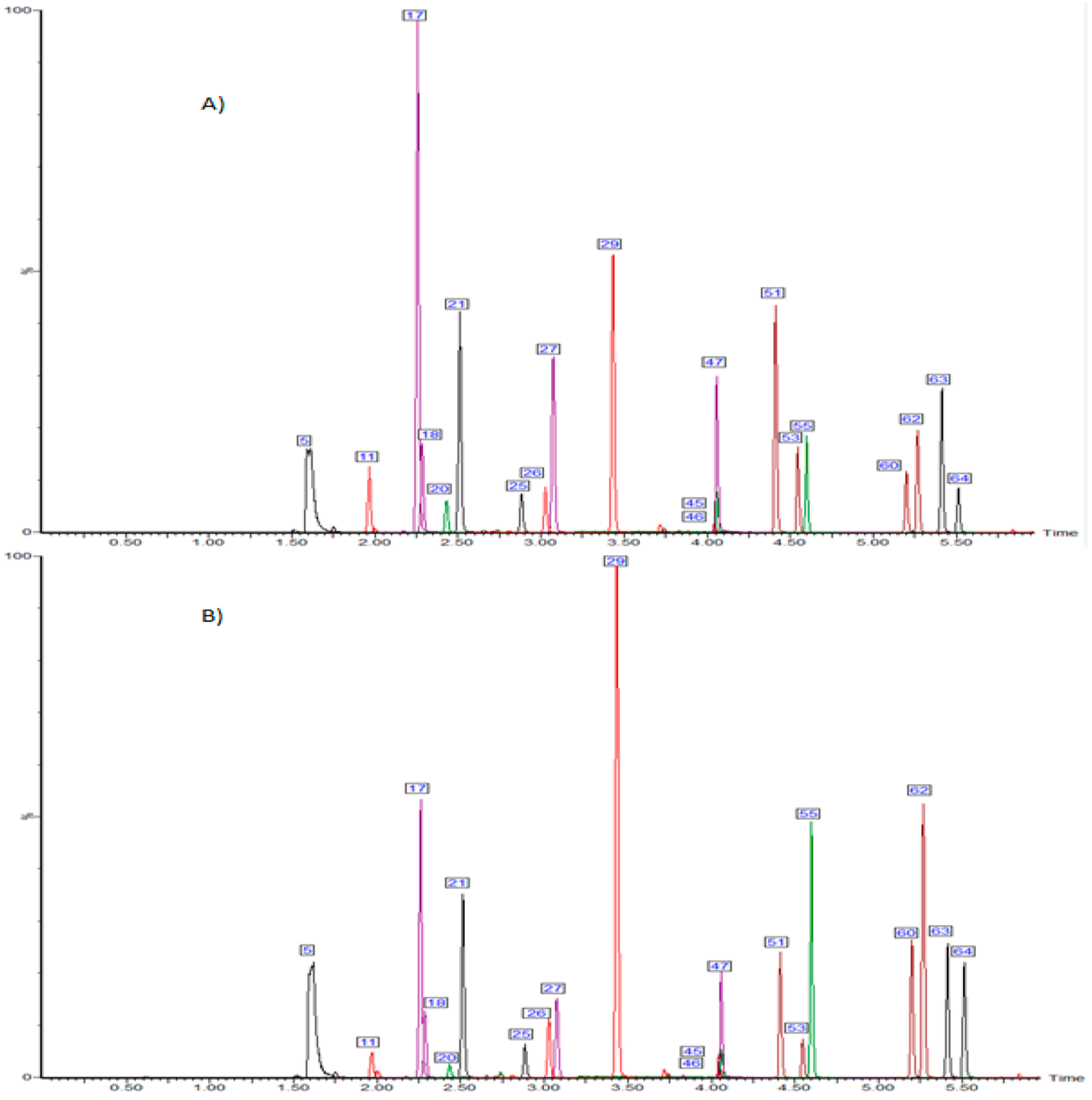

Figure 2. Scaled SRM chromatogram of an ALF (A) and a control (B) subject, respectively.

acid and 2-aminobutyric acid; isoleucine and leucine; however, all were resolved by the chromatographic method (illustrated in Figure S5).

Sample preparation was investigated on the basis of either protein precipitation with sulphosalicylic acid or various organic solvents (acetonitrile, methanol and isopropanol), with or without acidification using 1% v/v formic acid. This led to the selection solvent precipitation with two volumes of isopropanol, containing 1% v/v formic acid, to one of diluted plasma/serum sample based on better sensitivity and reduced matrix effects compared to, for example, methanol, sulphosalicylic acid, among others (data not shown).

Method Validation. Following the development of a suitable UHPLC-MS/MS system, the suitability of the method for the analysis of amino compounds was then evaluated for selected analytes (Table 1). In the absence of true biological blank samples, standard curves and QC samples were prepared in 50:50 water/methanol (v/v). A range of analytical attributes were investigated, including linearity, lower and upper limits of quantification (LLOQ, ULOQ), intra- and interday accuracy and precision, specificity, carryover, recovery, matrix and other interferences, and analyte and derivative stability. Standard curves were found to be linear over the range measured 1−400 μM, with correlation coefficients (R) of 0.993 or better as determined over the 3 days of the validation (Supplementary Data Table S2). The concentration range covered by the standard curve equates, in this instance, to 2−800 μM for plasma/serum due to the 1:1 dilution of the samples prior to

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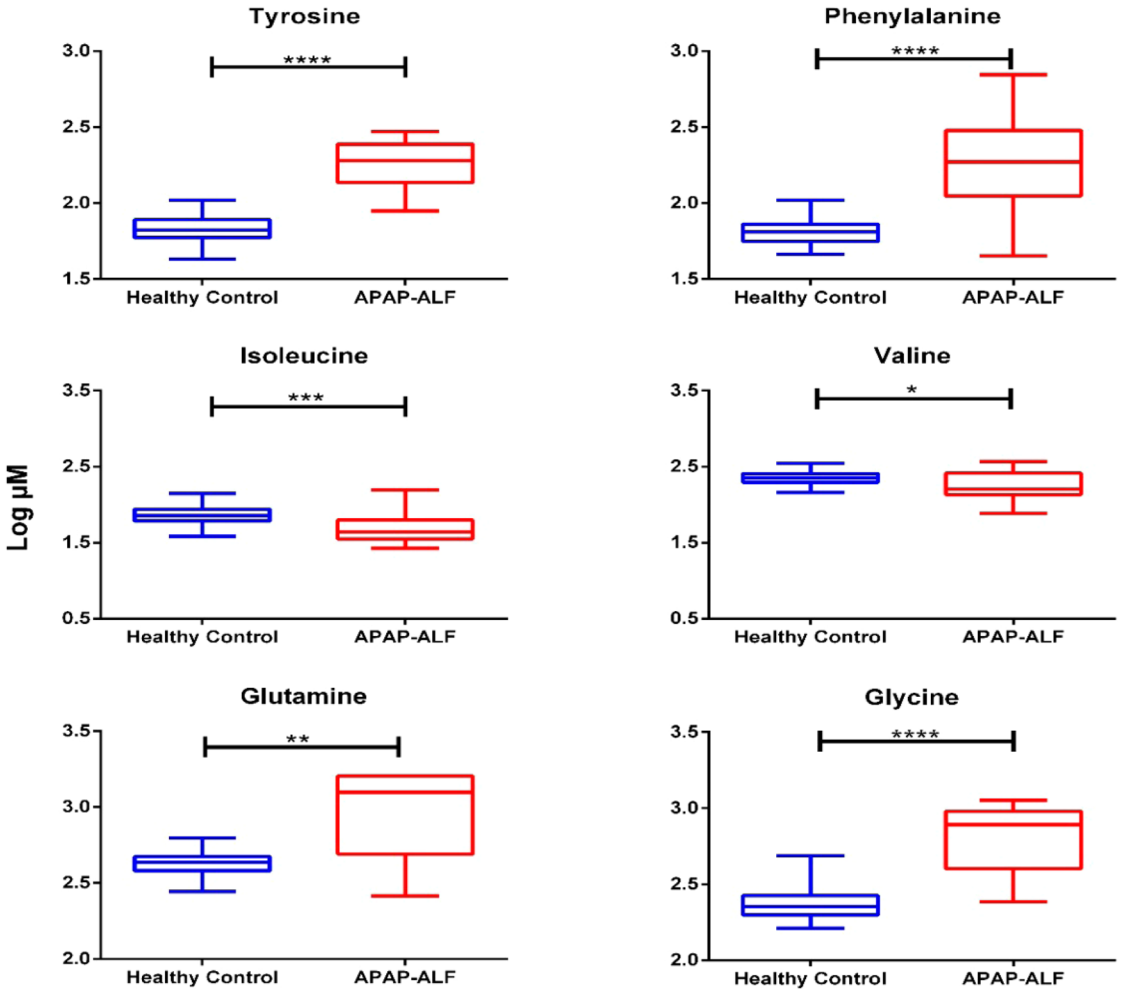

Figure 3. Amino acid concentrations (log μM) in control (blue) and ALF (red) subjects, box plots; bar, median; box, interquartile range; whiskers, upper and lower values. A two-tailed Mann−Whitney U test was applied to test for statistical significance, p-values for these are denoted by **** $p <$ 0.0001; *** < 0.001; ** < 0.01; * < 0.1.

protein precipitation. The underivatized analytes were stable for 6 h at ambient temperature or frozen at −20 °C for 48 h (Supplementary Data Tables S3 and S4, respectively). Similarly, the derivatized analytes were also stable at ambient temperature and in the autosampler at 4 °C for up to 1 week (1 week stability data are provided in Supplementary Tables S5 and S6, respectively). Carryover was 11% or less for all of the analytes tested, whereas that of the ISs was less than 1%.

The intra- and interday performance of the assay was evaluated by the analysis of samples on three separate occasions on three separate days over the concentration range of 2−800 μM, and the method was found to be reproducible and accurate. For both intra- and interday analysis a LLOQ of 2 μM was obtained for 18 of the amino acids (summarized in Table 1). A further 8 of the target analytes (ethanolamine, histidine, proline, serine, 2-aminoisobutyric acid, citrulline, and 2-aminobutyric acid and 4-aminobutyric acid (GABA)) gave acceptable results results using a LLOQ of 6 μM, whereas an additional 7 compounds (arginine, carnosine, glycine, 3-methylhistidine, 1-methylhistidine, hydroxylysine, and ornithine) could be quantified using LLOQs of 20 μM. The ULOQ for all analytes was 800 μM. A summary of the validated analytical ranges for these analytes is provided in Supplementary Table S7.

The intra- and interday precision of the assay was determined by examining the CVs of the QC standards for each of the analytes and ranged from 0.91 to 16.9% for the intraday determinations and 2.10−15.9% for the interday comparison. The corresponding figures for intra- and interday accuracy were 0.05−15.6 and 0.78−13.7%, respectively. Mean intra- and interday bias were calculated to be 0.04−16.4% and 0.04−12.5%, respectively. The mean interday concentration and precision data are provided in Table S8. Specificity, in terms of matrix and other interferences was assessed for matrix-to-analyte (at the LLOQ), matrix-to-internal standard, analyte-to-internal standard, and internal standard-to-analyte interference. Both matrix-to-analyte and matrix-to-internal standard were ≤20%. In the case of internal standard-to-analyte (at the LLOQ), the acceptance criterion of ≤20% was met in all cases except for glycine-d_5. This IS is effectively glycine-d_2 as 3 of the deuterium atoms are exchangeable with water protons on dissolution and, in this instance, the 2 mass unit difference between analyte and IS was insufficient to ensure specificity and resulted in internal standard-to-analyte interference. Replacement of glycine-d_5 with the alternative IS glycine-$^{13}C_2$ ^{15}N d_2 should eliminate this issue. All of the analytes gave values of ≤5% when analyte-to-IS interference was investigated except for glycine and glutamine.

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The stable-isotope-labeled compounds used as internal standards were used to assess gross matrix interferences following spiking into six different human plasma and serum samples. While differences were noted matrix matrix interferences were generally ≤20%.

Analyte recoveries, as determined using stable-isotope-labeled ISs spiked into plasma and serum, were high and repeatable (74−78 and 93−101% respectively at 3 μM) (Supplementary Table S9), as would be expected for a method with minimal sample preparation,

Application to Human Subjects. Following validation, the method was then applied to the analysis of samples obtained from patients suffering from acute liver failure and normal healthy controls. ALF is characterized by a rapid decline in liver function following a catastrophic insult to the liver (e.g., acetaminophen overdose or viral hepatitis). The liver plays a major role in amino acid metabolism and is central to the regulation of metabolic pathways.[53] In liver disease, these pathways are known to be affected by depletion of the branched chain amino acids, including valine, leucine, and isoleucine, and increased concentrations of aromatic amino acids, including phenylalanine and tyrosine (the Fischer ratio).[54] Perturbation of the urea cycle and a limited capacity for detoxification of ammonia with glutamine are associated with acute liver failure and in particular with hepatic encephalopathy, although the mechanism and prognostic significance remains unclear. Application of the methodology described above to plasma samples derived from healthy controls (n = 40) and patients with APAP-induced ALF (n = 14) revealed changes in 15 amino compounds that were subject to quantification. Changes were also noted in the relative amounts of aminoadipic acid, 3-aminopropanoic acid, 2-aminoisobutyric acid, cysteine, cystathionine, homoserine, histidine, hydroxylysine, 3-methylhistidine, and 4-aminobutyric acid (GABA) (Zia et al, in preparation). Selected ion chromatograms for a "typical" control and ALF subject are shown in Figure 2. As expected, significantly elevated concentrations of phenylalanine and tyrosine were observed in the ALF group with mean concentrations of phenylalanine in controls of 65 μM compared to 245 μM in ALF, whereas for tyrosine, the concentrations were 69 versus 191 μM in controls and patients, respectively (Figure 3). In the case of isoleucine, concentrations were reduced from 76 μM in control subjects to 56 μM in patients, whereas valine concentrations declined from 234 μM in controls to 188 μM in patients. Changes were also seen for glutamine and glycine, which were elevated in ALF, with the former rising from 427 μM to 1123 μM and the latter from 249 μM to 693 μM in patients (see Figure 3). Further results for quantifiable amino compounds are provided in Supplementary Figure S6. Effects were also noted on sarcosine with this analyte not detected in controls but observable with concentrations over the range 2.4−7.2 μM in ALF patients. Changes were also noted in the relative amounts of aminoadipic acid, β-alanine, β-amino-iso-butyric acid, cysteine, cystathionine, homoserine, histidine, hydroxylysine, 3-methylhistidine, and γ-amino-n-butyric acid; however, while detectable, these compounds were below the limits of quantification (data not shown). Furthermore, a 6-fold increase was observed for 5-aminovaleric acid in ALF patients relative to controls (compound 43, see Table 1), an amino compound which was subject to monitoring but not quantification in the current method. As well as confirming the known effects of ALF, such as the expected changes in aromatic and branched chain amino acids, the application of this UPLC-MS method has illustrated changes in the amounts of other amino-containing compounds in response to liver injury, including the previously unreported effects on plasma sarcosine concentrations.

CONCLUSIONS

The use of 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AccQTag UltraTM) for the targeted analysis of amino-containing compounds provides stable derivatives with good RPLC and MS properties. These derivatives enable the sensitive, reproducible and specific analysis of these compounds in human serum and plasma, requiring only 10 μL of sample, and the short analysis time makes the methodology attractive for high throughput targeted UPLC-ESI-MS/MS analysis. While confirming previous observations application of the method also revealed novel increases in sarcosine concentrations in patients with ALF as a result of APAP overdose. This methodology should be of great benefit in supporting metabolic phenotyping studies in epidemiological and clinical environments.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.6b04623.

Derivatization reaction between AccQTag Ultra and amines; mass spectra of cystine (a) and lysine (b) derivatives; UPLC separation of selected standards; separation of stable-isotope-labeled internal standards; resolution of the derivatives of isomeric amino acids and isobaric analytes; results for healthy controls and patients with APAP-induced ALF; sample treatment and derivatization for calibration standards/QCs, single blanks, plasma/serum, and double-blank samples; standard curve linearity data obtained during the 3 day validation; summary of short-term stock solution stability; freeze thaw stability of stock solutions; benchtop stability postderivatization; autosampler stability; summary of the valid concentration ranges for 38 analytes subject to quantification; interday validation data; recovery data for quantified analytes (PDF)

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Notes

The authors declare no competing financial interest.

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